REMARKS

Claims 25, 26, 43-46 and 69-73 are pending. Claims 43, 45, and 46 have been amended and claims 49, 57, 60, 61, 67 and 68 have been cancelled.

Applicants submit that no new matter has been added by way of the present submission. For instance, claim 43 has been amended to specifically define the product as ethanol as already recited claim 43 as previously pending. Claim 43 has also been amended to define the host microorganism as *Saccharomyces* spp. or *Schizosaccharomyces* spp., as supported by the specification at, for instance, page 7, lines 7-15. Also, claim 43 has been amended to specifically define the enzyme as NAD-dependent glutamate dehydrogenase and malic enzyme as supported by pending claim 43 and the present specification at page 7, lines 25-32, page 10, line 32 to page 11, line 14 and page 18, lines 11-12. Parallel amendments to other claims concerning the limitation to ethanol were also made. New claim 69 is supported by the present specification at page 1, lines 23-31 and page 2, lines 20-27. New claims 70 and 71 are supported by the present specification at, for example, page 7, lines 10-15. New claims 72 and 73 are supported by the present specification at, for example, page 13, line 29 to page 15, line 14. Accordingly, no new matter has been added.

In view of the following remarks, Applicants respectfully request that the Examiner withdraw all outstanding rejections and allow the currently pending claims.

Constructive Election

The Examiner maintains that a constructive election by original presentation exists only for the presently pending claims in as much as they relate to the production of "ethanol."

Although Applicants disagree with the Examiner, the claims have been limited to the production of ethanol. Accordingly, the claims do not encompass allegedly non-elected subject matter.

Issues Under 35 U.S.C. §112, first paragraph

The Examiner has rejected various claims pursuant to U.S.C. §112, first paragraph. The Examiner has based these rejections upon "written description" and "enablement". Applicants will separately address each of these rejections below.

1. Written Description

The Examiner has rejected claims 43, 45, 46, 49, 57, 60, 61, 67 and 68 under 35 U.S.C. §112, first paragraph as allegedly failing to satisfy the "written description" requirement. Applicants respectfully traverse.

First, the Examiner asserts that the claims are drawn to the production of "a product" utilizing an allegedly large genus of host cells of "any" yeast species transformed to express one or more of eight exogenous dehydrogenases and/or reductases. Thus, the Examiner asserts that there exists insufficient written description. Applicants respectfully disagree with the Examiner.

There exists written description in the present specification for more than just the specific transformants. The written description requirement does not require that Applicants disclose an exhaustive list of examples which cover every possible permutation of host cells, for instance a large number of host cells, as well as every permutation of dehydrogenase and/or reductase. In fact, to satisfy the written description requirement of 35 U.S.C. §112, first paragraph, a patent specification need simply describe the claimed invention in sufficient detail that one skilled in

the art can reasonably conclude that the inventor had possession of the claimed invention. Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991).

Applicants draw the Examiner's attention to the fact that the claims as pending specifically define the host microorganism as *Saccharomyces* spp. or *Schizosaccharomyces* spp.. Also, the claims recite ethanol as the specific product formed. Moreover, the claims recite the particular enzymes of NAD-dependent glutamate dehydrogenase and malic enzyme. As such, Applicants submit that present specification fully describes the presently claimed subject matter in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of the claimed invention at the time of filing.

Second, the Examiner has stated that the production of ethanol is dependent upon fermentation conditions and the genetic set of transformants used for fermentation. In particular, at page 9 of the outstanding Office Action the Examiner has referred to several references which allegedly indicate that the overexpression of certain dehydrogenases may in fact lead to a decrease in ethanol production. Applicants respectfully request that the Examiner reconsider this position.

The references cited by the Examiner show that overexpression of glutamate dehydrogenase (see Da Silva *et al.*, and Nissen *et al.*) or of glycerol-3-phosphate dehydrogenase (see Eglington *et al.*) may lead to a decrease in ethanol production. Or, the references show that deletion of glycerol-3-phosphate dehydrogenase increases ethanol production (see Valadi *et al.*).

Applicants are enclosing herewith another reference, Roca *et al.*, *Applied and Environmental Microbiology*, Vol. 69, No. 8, pp. 4732-36, August 2003. This article shows that

the deletion of GDH1 leads to improved ethanol production, but that simultaneous overexpression of GDH2 is necessary to recover the growth performance.

Applicants point out that the present claims do not recite glutamate dehydrogenases generically, but rather recite NAD-dependent glutamate dehydrogenase. However, even in view of the present claims, Applicants take this opportunity to stress that the expression of a dehydrogenase is performed to preferably create a pair of dehydrogenases. As explained in the present specification, for example at page 10, the pair of dehydrogenases have opposite coenzyme specificities for NAD/NADH and NADP/NADPH, but at least one common substrate. When both dehydrogenases are simultaneously expressed, for instance, in the cytosol, they are functionally coupled in that they catalyse reactions to equilibrate NAD/NADH and NADP/NADPH. As further explained, this leads to an increase in the production of, for example, ethanol.

Further, Applicants point out that the Examiner has incorrectly listed certain host strains. The correct listing of specific strains is as follows:

H1346: basic *S. cerevisiae* strain without XR or XDH or XK

H1469: H1346 + XR + XDH

H1803: H1469 + XK + GDH

H1805: H1469 + XK + (GDH) control plasmid

H1791: H1346 + GDH(no XR+XDH)

H1793: H1346 + (GDH) control plasmid

H2193: H1346 + MAE (no XR + XDH)

H2189: H1346 + (MAE) control plasmid

H2195: H1469 + MAE

H2191: H1469 + (MAE) control plasmid

H2217: H1469 + XK

H2222: H2217 + MAE

H2221: H2217 + (MAE) control plasmid

H2153: basic *Sch. pombe*

H2369: H2153 + XR + XDH + MAE

H2370: H2153 + XR + XDH + (MAE) control plasmid

A review of the above reveals that there are disclosed embodiments which produce a product but do not have either xylose reductase (XR) or xylitol dehydrogenase (XDH). Thus, claim 43 has not been so limited. As such, Applicants submit that the present claims fully satisfy the written description requirements of 35 U.S.C. § 112, first paragraph.

Third, the Examiner has also rejected claims 57-68 (Applicants point out that at the time of the Examiner's rejection, only claims 57, 60, 61, 67 and 68 were pending) as allegedly lacking a sufficient written description. However, Applicants submit that claims 57-68 are no longer pending, claims 57, 60, 61, 67 and 68 having been cancelled by this amendment. Thus, this rejection is moot.

Fourth, the Examiner rejects claims 46 and 68 asserting that the method of increasing the production of ethanol from a substrate of "carbohydrates" is not sufficiently described since Applicant allegedly discloses only the production of ethanol from glucose or xylose. Applicants disagree.

Although the present specification contains many references to substrates such as glucose and xylose, other substrates are also disclosed. For instance, the Examiner is referred to references to carbohydrates generally (see page 17, line 29 to page 18, line 14), pentoses (see page 15, lines 16-19, page 16, line 33 to page 17, line 6), and hexoses (see page 18, lines 22-27). Accordingly, even though some examples may be limited to glucose or xylose, the written description is not so limited. In fact, as evident from the specification, production of ethanol is possible from many different raw materials, including carbohydrates. Accordingly, this rejection is moot. Reconsideration and withdrawal thereof are respectfully requested.

2. Enablement

The Examiner has rejected claims 43, 45, 46, 49, 57, 60, 61, 67 and 68 under 35 U.S.C. §112, first paragraph for the reasons recited at pages 10-14 of the outstanding Office Action. Applicants respectfully traverse.

The Examiner points out that the nature of the invention encompasses transformation of "any" yeast cell (including any species of yeast, wild type, naturally occurring mutants as well as any mutants and transformants man-made) with the particularly claimed genes. Applicants traverse and submit that the present claims, for instance, claim 43, have been limited to the particular hosts of *Saccharomyces* spp. and *Schizosaccharomyces* spp., to the particular genes of NAD-dependent glutamate dehydrogenase and malic enzyme, and to the production of ethanol. Further, claims 57, 60, 61, 67 and 68 have been cancelled.

As discussed above, the present specification provides ample guidance concerning the production of ethanol by transforming *Saccharomyces* spp. or *Schizosaccharomyces* spp. with a

nucleotide sequence encoding one of NAD-dependent glutamate dehydrogenase and malic enzyme. Further, Applicants direct the Examiner's attention to dependent claims 70 and 71, which recite more specific host organisms. Based upon the above, Applicants respectfully submit that those of skill in the art would be able to make and use the presently claimed subject matter without undue experimentation. Thus, this rejection is moot. Reconsideration and withdrawal thereof are respectfully requested.

In summary, Applicants respectfully submit that the above remarks and/or amendments fully address and overcome the outstanding rejections. Accordingly, the Examiner is respectfully requested to withdraw all outstanding rejections and allow the currently pending claims.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact the undersigned at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicant(s) respectfully petition(s) for a three (3) month extension of time for filing a reply in connection with the present application, and the required fee is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

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Respectfully submitted,

By _____ #42,874

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Metabolic Engineering of Ammonium Assimilation in Xylose-Fermenting *Saccharomyces cerevisiae* Improves Ethanol Production

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Cofactor imbalance impedes xylose assimilation in *Saccharomyces cerevisiae* that has been metabolically engineered for xylose utilization. To improve cofactor use, we modified ammonia assimilation in recombinant *S. cerevisiae* by deleting *GDH1*, which encodes an NADPH-dependent glutamate dehydrogenase, and by overexpressing either *GDH2*, which encodes an NADH-dependent glutamate dehydrogenase, or *GLT1* and *GLN1*, which encode the GS-GOGAT complex. Overexpression of *GDH2* increased ethanol yield from 0.43 to 0.51 mol of carbon (Cmol) Cmol^{-1}, mainly by reducing xylitol excretion by 44%. Overexpression of the GS-GOGAT complex did not improve conversion of xylose to ethanol during batch cultivation, but it increased ethanol yield by 16% in carbon-limited continuous cultivation at a low dilution rate.

In order to develop an efficient process for the production of bioethanol from lignocellulosic material, there have been many attempts to improve the conversion of xylose to ethanol by construction of recombinant *Saccharomyces cerevisiae* strains. Even though the open reading frames encoding the three first enzymes involved in xylose metabolism, i.e., xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulose kinase (XK), have been identified in the genome of *S. cerevisiae* (10, 15), the genes are poorly expressed (or not expressed at all), and xylose cannot be metabolized without introduction of heterologous genes from naturally xylose-fermenting microorganisms and overexpression of endogenous XK (3, 6, 17). In the resulting strains, there is a low rate of xylose consumption and substantial xylitol secretion.

Since xylose-metabolizing strains of *S. cerevisiae* harbor a NAD(P)H-dependent xylose reductase and a NAD$^+$-dependent xylitol dehydrogenase from *Pichia stipitis*, one of the main reasons for xylitol excretion can be explained by the redox imbalance occurring at the level of XR and XDH. Metabolic engineering of redox metabolism may therefore be a strategy for improving the conversion of xylose to ethanol. This has been illustrated by deletion of the *zwf1* gene encoding glucose-6-phosphate dehydrogenase in a xylose-metabolizing strain of *S. cerevisiae* (7). The main source of NADPH originating from the oxidative part of the pentose phosphate pathway has thereby been reduced, and during growth on xylose, this has resulted in a significant improvement of ethanol yield, from 0.31 g g^{-1} (yield on consumed sugar) to 0.41 g g^{-1} with a concomitant reduction in the xylitol yield.

In this study, we have taken a different approach to modulating the redox metabolism to favor xylose metabolism, namely through metabolic engineering of the ammonia assimilation. Ammonium assimilation in *S. cerevisiae* involves glutamate dehydrogenase and glutamine synthetase. Glutamate dehydrogenase catalyzes the synthesis of glutamate from ammonium and 2-ketoglutarate. The most important enzyme for ammonia assimilation is the NADPH-dependent glutamate dehydrogenase 1, encoded by *GDH1*. The other glutamate dehydrogenase that is present in *S. cerevisiae* is NADH dependent and is encoded by *GDH2*. This enzyme is usually catalyzing the degradation of glutamate into 2-ketoglutarate and ammonium (8). Coaction of two other enzymes, glutamate synthase (*GLT1*) and glutamine synthetase (*GLN1*), known as the GS-GOGAT system, may also assimilate ammonia into glutamate, using NADH as a cofactor and ATP. Nissen et al. (9) have shown that deletion of *GDH1* in *S. cerevisiae* resulted in an increased ethanol yield, concomitant with a decreased glycerol yield. This was due to a shift from use of NADPH to use of NADH in connection with ammonia assimilation, and hereby NADH generated in connection with biomass formation could be balanced through ammonia assimilation instead of through formation of glycerol. However, the specific growth rate was dramatically reduced, and overexpression of either *GDH2* or the GS-GOGAT system was necessary to recover the growth performance. Since this strategy decreases the utilization of NADPH in ammonia assimilation, more NADPH will be expected to be available for the reduction of xylose to xylitol.

In the present study, we therefore overexpressed *GDH2* or *GLN1* and *GLT1* in a strain with a deletion of *gdh1*. The *XYL1*, *XYL2*, and *XKS1* genes were introduced in each of these strains, resulting in xylose-fermenting strains. Batch and chemostat cultivations were carried out in order to investigate the physiology of the recombinant strains and to analyze the effect of redox balance modification on xylose metabolism.

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MATERIALS AND METHODS

Strains. All *S. cerevisiae* strains used in this study were derived from the CEN.PK 113-7D wild-type strain (Table 1). The genes encoding XR and XDH

TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	Origin/reference
CEN.PK 113-7D	*MATα SUC2 MAL2-8^C*	SRD Gmbh
TMB3001	*MATα SUC2 MAL2-8^C pADH-XYL1 pPGK-XYL2 pPGK-XKS1*	3
CPB.CR1	*MATα SUC2 MAL2-8^C pADH-XYL1 pPGK-XYL2 pPGK-XKS1 gdh1Δ*	This study
CPB.CR4	*MATα SUC2 MAL2-8^C pADH-XYL1 pPGK-XYL2 pPGK-XKS1 gdh1Δ pPGK-GDH2*	This study
CPB.CR5	*MATα SUC2 MAL2-8^C pADH-XYL1 pPGK-XYL2 pPGK-XKS1 gdh1Δ pPGK-GLT1 pPGK-GLN1*	This study

from *P. stipitis* and the endogenous gene for XK have already been integrated into the chromosome of CEN.PK 113-7D, using the integrative plasmid YipXR/XDH/XK, leading to the stable construct TMB3001 (3). The *GDH1* gene has been deleted using the *loxP-kanMX-loxP* disruption cassette (5). *GDH2*, *GLN1*, and *GLT1* have been put under a *PGK* constitutive promoter. Transformation with the plasmid YipXR/XDH/XK was performed using the lithium acetate method as described by Gietz et al. (4). The strains were stored at 4°C on yeast extract-peptone-dextrose agar plates.

Medium preparation. A defined medium containing trace metal elements and vitamins was used in all cultivations. Fatty acids in the form of Tween 80 and ergosterol were added to the cultivations to sustain anaerobic growth of *S. cerevisiae*. The medium used for batch and chemostat cultivations was prepared according to the method of Verduyn et al. (18). For cultivation on glucose, the concentration was 20 g liter^{-1}; for mixed sugar cultivations, 20 g of glucose liter^{-1} and 50 g of xylose liter^{-1} were used.

Batch cultivation. Cultivations were carried out in well-controlled four-baffled 5-liter in-house-manufactured bioreactors with a working volume of 4 liters, and the temperature was controlled at 30°C. The bioreactors were equipped with two disk-turbine impellers rotating at 500 rpm. The pH was kept constant at 5.0 by automatic addition of 2 M NaOH. Nitrogen containing less than 5-ppm O_2 (AGA, Copenhagen, Denmark) was used for sparging of the fermentor at 0.2 vvm. Batch cultivations were performed in duplicate or triplicate in order to evaluate the reproducibility of the experiments. A mean value was calculated for all the parameters, as well as the standard deviation.

Continuous cultivation. Carbon-limited cultivations were carried out in 2-liter Applikon (Schiedam, The Netherlands) bioreactors with a constant working volume of 1 liter at 30°C and with a stirring speed of 500 rpm. The pH was kept constant at 5.0 by automatic addition of 1 M NaOH. Nitrogen containing less than 5 ppm of O_2 was sparged through the fermentor at 0.2 vvm. As batch cultivations, chemostat cultivations were performed in duplicate or triplicate in order to evaluate the reproducibility of the experiments.

Off gas analysis. Carbon dioxide and oxygen concentrations in the exhaust gas were determined by a Brüel and Kjær (Nærum, Denmark) 1308 acoustic gas analyzer (2).

Cell mass determination. The dry weight was determined as previously described (14). Biomass composition used in yield calculation was $CH_{1.8}O_{0.5}N_{0.2}$.

Analysis of extracellular metabolites. Samples taken from the cultivation broth were immediately filtered through a 0.45-μm-pore-size cellulose acetate filter (Osmonics) and stored at −20°C until analysis. Glucose, xylose, xylitol, glycerol, ethanol, succinate, and acetate concentrations were determined as previously described (14).

RESULTS

Batch cultivation. Physiology of xylose-fermenting *S. cerevisiae* strains CPB.CR1 (Δ*gdh1*), CPB.CR4 (Δ*gdh1 GDH2*), and CPB.CR5 (Δ*gdh1 GS-GOGAT*) in comparison with the parent type strain, TMB3001, was investigated during anaerobic batch cultivations on 20 g of glucose liter^{-1} or a mixture of 20 g of glucose liter^{-1} and 50 g of xylose liter^{-1}. Deletion of *gdh1* in CPB.CR1 resulted in a decrease in the specific growth rate to 0.16 (±0.002) h^{-1} compared with 0.34 (±0.006) h^{-1} for the parental strain TMB3001 during batch cultivation on glucose (Table 2). Overexpression of *GDH2* or the GS-GOGAT system in the *gdh1* deletion background resulted in a recovery of the specific growth rate to 0.32 (±0.002) h^{-1}. Deletion of *gdh1* led to a 48% decrease in the glycerol yield during batch cultivation on glucose down to 0.057 (±0.001) mol of carbon (Cmol) Cmol^{-1}. Similarly, the glycerol yield in CPB.CR4 and CPB.CR5 decreased to 0.06 (±0.004) and 0.07 (±0.001) Cmol Cmol^{-1}, respectively, compared to 0.11 (±0.003) Cmol Cmol^{-1} in TMB3001. Nevertheless, *gdh1* deletion alone did not improve ethanol yield, but it decreased to 0.41 (±0.006) Cmol Cmol^{-1} compared to 0.53 (±0.01) Cmol Cmol^{-1} for the parent type. For CPB.CR4, the increase in ethanol yield (0.56 ± 0.01 Cmol Cmol^{-1}) was 5%, whereas for CPB.CR5 the increase was not significant compared to the level in the parental strain (Table 2).

When cells were grown on a mixture of glucose and xylose, the glucose consumption rate decreased compared with growth on glucose alone (Table 2). Similarly, the specific growth rates were generally lower on the sugar mixture than with glucose as the sole carbon source. In CPB.CR1 as well as in CPB.CR5, the total sugar consumption rate decreased to 86 (±6.2) to 88 (±8.4) Cmmol of sugar g (dry weight)$^{-1}$ h^{-1} compared to 149 (±11) Cmmol of sugar g (dry weight)$^{-1}$ h^{-1} in TMB3001. On the contrary, overexpression of *GHD2* (CPB.CR4) increased the total sugar consumption rate by 20% up to 180 (±12) Cmmol g (dry weight)$^{-1}$ h^{-1}. As illustrated in Fig. 1, xylose was consumed much faster by CPB.CR4 than by TMB3001 in the initial phase of xylose consumption. We calculated the maximum xylose consumption rate at the time of glucose depletion. It increased from 13 (±0.6) in TMB3001 to 15 (±0.9) Cmmol g (dry weight)$^{-1}$ h^{-1} in CPB.CR4 (Table 2). After glucose depletion, the specific xylose consumption rate slowly decreased, and no difference of the xylose consumption rate could then be observed between the two strains after 100 h. Overexpression of the GS-GOGAT complex did not affect the maximum rate of xylose consumption during batch cultivation on a mixture of glucose and xylose. The glycerol yield was higher in CPB.CR1 (0.13 ± 0.005 Cmol Cmol^{-1}) and CPB.CR4 (0.10 ± 0.002 Cmol Cmol^{-1}) but was lower in CPB.CR5 (0.05 ± 0.001 Cmol Cmol^{-1}) than the parental strain, TMB3001 (0.07 ± 0.001 Cmol Cmol^{-1}) (Table 2). An 18% increase in the ethanol yield was achieved during batch cultivation of glucose and xylose by overexpression of *GDH2* and deletion of *GHD1* in CPB.CR4 from 0.43 (±0.01) to 0.51 (±0.01) Cmol Cmol^{-1}, with a concomitant decrease in the xylitol yield from 0.29 (±0.006) Cmol Cmol^{-1} down to 0.16 (±0.005) Cmol Cmol^{-1}, whereas the two other constructs had lower ethanol yields than the parental strain (Table 2).

Chemostat cultivation. Chemostat cultivations of the metabolically engineered strains were performed in order to analyze the metabolism of the strains at low sugar consumption rates, with the dilution rate set to 0.05 h^{-1}, i.e., the specific growth rate was 0.05 h^{-1}. The carbon source was either 20 g of glucose liter^{-1} or a mixture of 20 g of glucose liter^{-1} and 50 g of xylose liter^{-1} as during batch cultivation. As for the batch cultivation,

TABLE 2. Specific sugar consumption rates, specific growth rates, and product yield coefficients for anaerobic batch fermentations

Strain	Concn (g/liter) of carbon source(s)[l]	μ[a]	r_{glu}[b]	r_{xyl}[c]	Y_{SX}[d,e]	Y_{SG}[d,f]	Y_{SE}[d,g]	Y_{SC}[d,h]	Y_{Sxol}[d,i]	Y_{Xylxol}[d,j]
TMB3001	20 glu	0.34	150		0.10	0.11	0.53	0.20		
	20 glu + 50 xyl	0.27[a]	136	13	0.04	0.07	0.43	0.19	0.29	0.47
CBP.CR1 (Δ*gdh1*)	20 glu	0.16	130		0.07	0.057	0.41	0.22		
	20 glu + 50 xyl	0.12[a]	77	9.3	0.03	0.13	0.21	ND[k]	0.12	0.21
CPB.CR4 (Δ*gdh1 GDH2*)	20 glu	0.32	160		0.10	0.06	0.56	0.24		
	20 glu + 50 xyl	0.25[a]	166	15	0.03	0.10	0.51	0.19	0.16	0.26
CPB.CR5 (Δ*gdh1 GS-GOGAT*)	20 glu	0.32	140		0.09	0.07	0.54	0.23		
	20 glu + 50 xyl	0.24[a]	75	13	0.04	0.054	0.36	0.17	0.35	0.51

[a] Specific growth rate (h^{-1}) during first phase of growth on glucose, when glucose is used together with a smaller fraction of xylose.
[b] Specific consumption rate of glucose (Cmmol/gDW · h).
[c] Specific consumption rate of xylose (Cmmol/gDW · h) at the time when glucose was depleted.
[d] Yields (Y) are based on total consumed sugars.
[e] Yield for biomass (Cmol/Cmol).
[f] Yield for glycerol (Cmol/Cmol).
[g] Yield for ethanol (Cmol/Cmol).
[h] Yield for CO_2 (Cmol/Cmol).
[i] Yield for xylitol (Cmol/Cmol).
[j] Xylitol yield based on consumed xylose only (Cmol/Cmol).
[k] ND, not determined.
[l] glu, glucose; xyl, xylose.

glycerol yield on glucose decreased from 0.07 (±0.001) Cmol Cmol^{-1} in TMB3001 down to 0.05 (±0.002), 0.06 (±0.001), and 0.05 (±0.001) Cmol Cmol^{-1} in CPB.CR1, CPB.CR4, and CPB.CR5, respectively. The ethanol yield was improved in all the redox engineered strains, where the ethanol yield increased maximally with 14% in CPB.CR1 to 0.58 (±0.009) Cmol Cmol^{-1}, compared to 0.51 (±0.012) Cmol Cmol^{-1} in the parental strain (Table 3).

When cells were grown on a mixture of glucose and xylose, they all presented a biomass yield in the range of 0.07 to 0.09 Cmol Cmol^{-1} (Table 3). The glycerol yield was lower in CPB.CR1 and CPB.CR4, 0.02 (±0.001) and 0.03 (±0.001) Cmol Cmol^{-1}, respectively, whereas it was higher, 0.08 (±0.002) Cmol Cmol^{-1} in CPB.CR5, compared to 0.06 (±0.001) Cmol Cmol^{-1} in TMB3001. The ethanol yield during growth on the glucose-xylose mixture was higher in the three redox engineered strains, i.e., 0.40 (±0.01), 0.44 (±0.02), and 0.43 (±0.023) Cmol Cmol^{-1} in CPB.CR1, CPB.CR4, and CPB.CR5, respectively, compared to 0.37 (±0.015) Cmol Cmol^{-1} in the parental strain. Consequently, the xylitol yield was lower, 0.22 (±0.006), 0.19 (±0.005) and 0.17 (±0.01) Cmol Cmol^{-1} for CPB.CR1, CPB.CR4, and CPB.CR5, respectively. Nevertheless, the xylose consumption rate was lower for the *gdh1* deletion strain than for TMB3001. Overexpression of *GDH2* resulted in an increased xylose consumption rate compared with that of the *gdh1* deletion strain, but the specific xylose consumption rate was the same in TMB3001 and CPB.CR4, with 10.2 (±0.51) and 10.3 (±0.41) Cmmol g (dry weight)$^{-1}$ h^{-1}, respectively. However, contrary to what was found during the batch cultivations, overexpression of the GS-GOGAT system in a *gdh1* deletion background enhanced the xylose consumption rate from 10.2 (±0.51) to 11.4 (±0.52) Cmmol g (dry weight)$^{-1}$ h^{-1} (Table 3).

DISCUSSION

In this study, we showed that metabolic engineering of the ammonia assimilation in xylose-fermenting *S. cerevisiae* resulted in a substantially improved conversion of xylose to ethanol during anaerobic batch growth on glucose and xylose mixture and particularly that the formation of the by-product xylitol could be reduced by 44% in *S. cerevisiae* CPB.CR4 (Table 2). From analysis of the different recombinant strains in batch and chemostat cultures, the different results obtained between the engineered strains could basically be explained by two effects: (i) the modification of the redox metabolism in the recombinant strains and (ii) an increased use of ATP in the recombinant strain CPB.CR5 with overexpression of the GS-GOGAT pathway.

In the *S. cerevisiae* CPB.CR4 strain, NADH is used in ammonia assimilation due to the overexpression of *GDH2*, resulting in an improved availability of NAD^+ for the XDH in the xylose metabolism. Hereby, there is an increased conversion of xylitol to xylulose (44% lower xylitol yield than that of the parent strain). Furthermore, with the shift in cofactor use in the ammonia assimilation, NADPH is no longer used for glutamate synthesis and can be dedicated for use by the XR in the conversion of xylose to xylitol. Thus, xylose conversion was favored, and the consumption rate consequently increased by 15% compared to that of the parental strain TMB3001.

For CPB.CR5 with overexpression of the GS-GOGAT complex, there was an increased drain of ATP for ammonia assimilation. Since 1 mol of ATP is used to convert 1 mol of 2-ketoglutarate by the GS-GOGAT system, the ATP demand for growth is higher in CPB.CR5 than in CPB.CR1 and CPB.CR4, where only the cofactor use was modified. The intracellular ATP concentration has been reported in an *S. cerevisiae* strain to be as low as 1.78 mM where XK has been overexpressed during anaerobic fed-batch cultivation on xylose (17). These cultivation conditions are close to the ones prevailing during the batch cultivation of CPB.CR5. Therefore, one can assume that intracellular ATP concentration in CPB.CR5 is below 1.78 mM, since both XK and GS-GOGAT are overexpressed, and even below the affinity of ATP for XK, which has been reported to be around 1.5 mM (11). Hence, XK cannot operate efficiently, and xylulose phosphorylation is not sufficient to

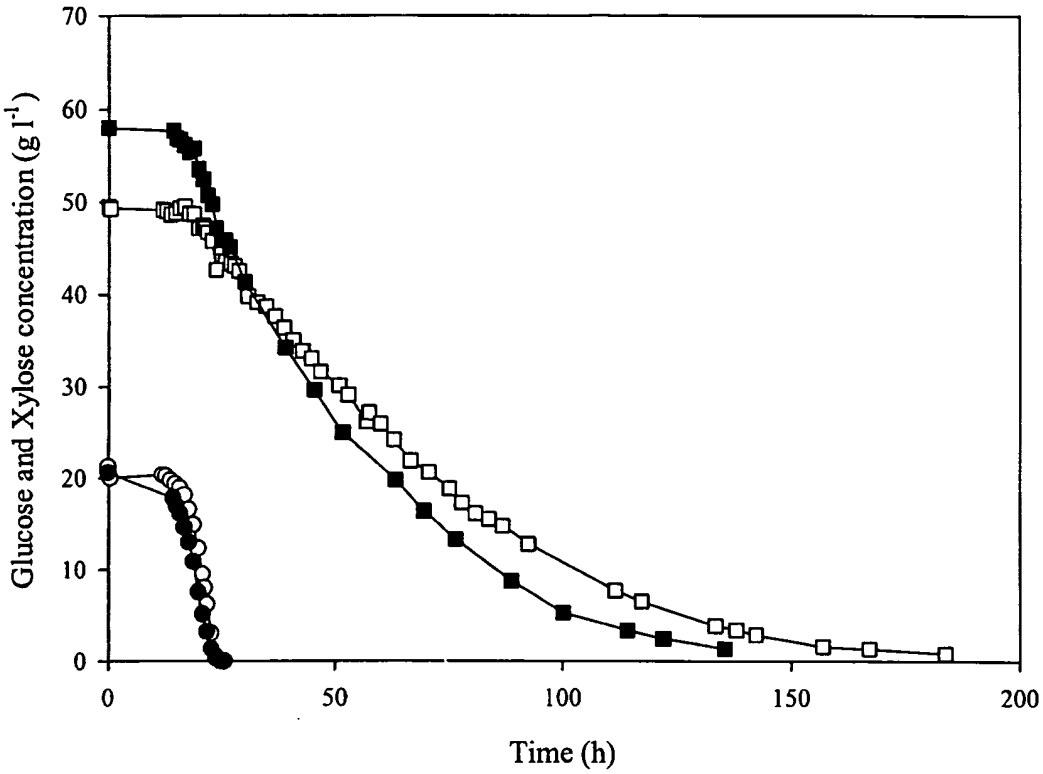

FIG. 1. Sugar consumption during batch cultivation on a mixture of glucose and xylose (glucose, ○ and ●; xylose, □ and ■) of parent type TMB3001 (open symbols) and CPB.CR4 (filled symbols).

make the downstream conversion of xylitol favorable (thermodynamics of XR and XDH favor formation of xylitol) (12, 13). Consequently, 20% more xylitol was produced than was the case with TMB3001. During chemostat cultures (Table 3), we find that in all the redox-engineered strains, ethanol yield from glucose and xylose was improved. In *S. cerevisiae* CPB.CR5, the xylose consumption rate increased by 15% and the xylitol excretion was 25% lower than in the parental strain, TMB3001. During carbon-limited chemostat cultivations at a low dilution rate, ATP levels are more elevated than during batch cultivations (16), and the kinase effect, originating from the coaction of GS-GOGAT and XK, was thereby lower than during batch cultivations, explaining why xylose conversion was enhanced in CPB.CR5.

In conclusion, engineering of ammonium assimilation made it possible to modify the redox balance in xylose fermenting *S.*

TABLE 3. Specific sugar consumption rates and product yield coefficients for anaerobic carbon-limited chemostat fermentations at a dilution rate of $D = 0.05\ h^{-1}$

Strain	Concn (g/liter) of carbon source(s)	r_{glu}[a] Cmmol/gDW · h	r_{xyl}[b] Cmmol/gDW · h	Y_{SX}[c] (Cmol/Cmol)	Y_{SG}[d] (Cmol/Cmol)	Y_{SE}[e] (Cmol/Cmol)	Y_{SC}[f] (Cmol/Cmol)	Y_{Sxol}[g] (Cmol/Cmol)	Y_{Xyltol}[h] (Cmol/Cmol)
TMB3001	20 glucose	19.6		0.11	0.07	0.51	0.29		
	20 glucose + 50 xylose	13.9	10.2	0.08	0.06	0.37	0.27	0.23	0.55
CPB.CR1 (Δ*gdh1*)	20 glucose	22		0.09	0.05	0.58	ND[i]		
	20 glucose + 50 xylose	15.9	8.3	0.09	0.02	0.40	0.31	0.22	0.58
CPB.CR4 (Δ*gdh1 GDH2*)	20 glucose	17.1		0.12	0.06	0.53	0.27		
	20 glucose + 50 xylose	15.6	10.3	0.07	0.03	0.44	0.23	0.19	0.46
CPB.CR5 (Δ*gdh1 GS-GOGAT*)	20 glucose	16		0.12	0.055	0.52	0.28		
	20 glucose + 50 xylose	15	11.4	0.07	0.08	0.43	0.23	0.17	0.43

[a] Specific consumption rate of glucose.
[b] Specific consumption rate of xylose.
[c] Yields (Y) are based on total consumed sugars. Yield for biomass.
[d] Yield for glycerol.
[e] Yield for ethanol.
[f] Yield for CO_2.
[g] Yield for xylitol.
[h] Xylitol yield based on consumed xylose.
[i] ND, not determined.

cerevisiae. Previous work on the modification of cofactors utilization resulted either in a decreased xylitol yield concomitant with an alteration of the xylose consumption rate (7) or in an increased xylose consumption rate accompanied with an increased xylitol excretion (1). In this study, it was possible to improve both the xylose consumption rate and the ethanol yield from xylose by deletion of *GDH1* and overexpression of either *GDH2* or both *GLT1* and *GLN1*. This confirms the importance of balanced cofactor use in xylose metabolism.

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